PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 5/00 | A2

(11) International Publication Number: WO 00/23567

(43) International Publication Date: 27 April 2000 (27.04.00)

(21) International Application Number: PCT/EP99/07741

(22) International Filing Date: 14 October 1999 (14.10.99)

(30) Priority Data:

09/173,633	16 October 1998 (16.10.98)	US
60/132,476	4 May 1999 (04.05.99)	US

(71) Applicant *(for all designated States except AT US)*: NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, CH–4058 Basel (CH).

(71) Applicant *(for AT only)*: NOVARTIS–ERFINDUNGEN VERWALTUNGSGESELLSCHAFT M.B.H. [AT/AT]; Brunner Strasse 59, A–1230 Vienna (AT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: LAVAU, Catherine, P. [FR/US]; 4296–A Wilkie Way, Palo Alto, CA 94306 (US). YOUNG, Judy, Carol [US/US]; 2833 Tramanto Drive, San Carlos, CA 94070 (US). HILL, Beth, Louise [US/US]; 981 Kintyre Way, Sunnyvale, CA 94087 (US).

(74) Agent: BECKER, Konrad; Novartis AG, Corporate Intellectual Property, Patent & Trademark Department, CH–4002 Basel (CH).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: PROMOTION OF SELF–RENEWAL AND IMPROVED GENE TRANSDUCTION OF HEMATOPOIETIC STEM CELLS BY HISTONE DEACETYLASE INHIBITORS

(57) Abstract

A method of promoting stem cell self–renewal is disclosed which comprises exposing a population of stem cells, particularly hematopoietic stem cells, to an effective dose of a histone deacetylase inhibitor, particularly trichostatin A, trapoxin, or chlamydocin. The invention is also directed to the use of histone deacetylase inhibitors to increase the number of transduced stem cells.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

PROMOTION OF SELF-RENEWAL AND IMPROVED GENE TRANSDUCTION OF HEMATOPOIETIC STEM CELLS BY HISTONE DEACETYLASE INHIBITORS

This invention relates to the field of self-renewal divisions and transduction of stem cells, particularly hematopoietic stem cells (HSCs).

The maintenance of the hematopoietic system relies on primitive pluripotent hematopoietic stem cells that have the capacity to self-renew and repopulate all the blood cell lineages with relevant progenitor cells. Due to their capacity for self-renewal and their pluripotent, long term reconstituting potential, HSCs have long been considered ideal for transplantation to reconstitute the hematopoietic system after treatment for various hematologic disorders or as a target for the delivery of therapeutic genes. Additionally, human HSCs have potential applications in restoring the immune system in autoimmune diseases and in the induction of tolerance for allogenic solid organ transplantation.

Early in hematopoiesis, a pluripotent stem cell differentiates and gives rise to either lymphoid, myeloid, or erythroid restricted cells. These cells in turn differentiate into progenitor cells that are committed to a specific cell type. Evidence indicates these progenitor cells have lost the capacity for self-renewal and contribute little if anything to engraftment following transplantation. It appears that infused HSCs are necessary for short term and sustained engraftment. Furthermore, the kinetics of engraftment is proportional to the number of infused HSCs. This is particularly true in allotransplantation settings. Therefore attempts to improve graft quality may be accomplished if there is an increase in HSC number. Additionally, increase in HSC number by culturing *in vitro* will allow improved therapeutic approaches for the treatment of many diseases including cancer, autoimmune and certain genetic diseases.

HSCs are generally obtained from bone marrow, mobilized peripheral blood and umbilical cord blood. Presently large volumes of bone marrow must be extracted and frozen

to ensure that sufficient number of stem cells are present to reconstitute the hematopoietic system. Additionally, single cord blood donors provide insufficient numbers of hematopoietic stem cells for adult therapeutic use. Therefore, methods developed for promotion of HSC self-renewal as distinguished from stem cell expansion could provide a tremendous advantage for therapeutic uses.

Since the 1980's, numerous discoveries have been made concerning the influence of growth factors, such as interleukins and cytokines, on hematopoietic cells. These hormones exert a profound effect on the growth and differentiation of hematopoietic cells. Some of these molecules include interleukin (IL) –1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, erythropoietin (EPO), granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), and granulocyte-macrophage-colony stimulating factor (GM-CSF). In an attempt to achieve optimal production of progenitors during *in vitro* culture, these factors have been tested in many combinations. A number of major breakthroughs in stem cell replication came with the discovery of c-kit ligand and a ligand for the fetal liver kinase receptor, flk2/flk3 ligand (FL). Both of these cytokines synergize with other cytokines *in vitro* and have the effect of sustaining viability and recruiting very primitive hematopoietic cells into cycle without increasing their differentiation. Additionally, the molecule thrombopoietin (TPO) which promotes megakaryocytopoiesis was discovered to have a potent effect on the viability and replication of both human and murine primitive hematopoietic progenitor cells *in vitro*. Other culture conditions and additives have been studied with the hope of optimizing HSC replication. These aspects of the culture system include the culture media, the surface for adhesion, and schedules for replacement of gases and nutrients. The role of stroma cells has also been investigated in stem cell self-renewal technology. Cell adhesion molecules expressed on the surface of primitive cells, such as VLA-4, VLA-5 and L-selectin have been explored for effects on stem cell cycling. Specific receptor-ligand interactions between stromal cells and HSCs have been identified that appear to be critical for stem cell maintenance.

While the manipulation of culture conditions is one approach to promote HSC self-renewal, the present invention is concerned with the transcriptional regulation of genes

involved in self-renewal through the modulation of histone acetylation.

In eukaryotic cells, DNA molecules are tightly complexed with histone proteins to form chromatin, and changes in chromatin structure are known to be of fundamental importance in regulation of gene expression. Histones are rich in arginine and/or lysine, and are classified in five major groups designated H1(lysine-rich), H2A and H2B (slightly lysine rich), and H3 and H4 (arginine rich). These proteins have two domains, the histone fold domain and the amino tail domain. The amino tail domain interacts with DNA at specific sites between the positively charged arginine/lysine residues and the negatively charged phosphate groups of the DNA. At specific points in the cell cycle, the amino tail domain undergoes transient modifications including acetylation. The histone-modifying enzymes, particularly acetyltransferases and deacetylases, are known to play active roles in the transcription and assembly of chromatin. While not meant to be limiting, the prevailing scientific view is that a correlation exists between the level of histone acetylation and deacetylation and transcriptional activity, and that histone acetylation leads to a decondensation of chromatin which facilitates the access of transcriptional factors and components of the basal transcriptional machinery to DNA. (Grunstein, Michael, *Nature* 389:349 - 352, (1997); Wade, P. et al., *TIBS* 22:128 - 132, (1997)). It is also believed that some transcriptional cofactors (activators) have histone acetyl transferase activity, and therefore these coactivators could direct local destabilization of repressive histone-DNA interactions.

Conversely, a growing number of transcriptional repressors have been found to act through the recruitment of histone deacetylases (HDACs). Several repressors of transcription are involved in the mechanisms that govern cellular differentiation and proliferation and have been implicated in normal hematopoiesis or leukemogenesis. Some of these repressors are briefly mentioned.

Retinoid receptors are involved in myeloid maturation but may also play a critical role in the development of pluripotent hematopoietic stem cells (Tsai, et al. *Genes & Dev.* 8: 2831

(1994)). While these receptors behave as transcriptional activators when bound to their ligand, in the non-ligand state they repress the basal transcriptional activity by associating with the transcriptional corepressors SMRT and N-CoR. Nagy, et al. has shown that the SMRT transcriptional corepressor exerts its activity by forming a multisubunit repressor complex in which the histone deacetylase 1 (HDAC1) is recruited. (Nagy, et al. *Cell,* 89: 373 – 380 (1997)) It was confirmed that histone deacetylation is fundamental for retinoid signaling by showing that the deacetylase inhibitor trichostatin A (TSA) could greatly enhance the biological activity of retinoic acid. This was demonstrated using the HL60 promyelocytic cell line that undergoes myeloid maturation in response to retinoic acid. In the presence of sub-optimal doses of retinoic acid, a full differentiative response of the cells was achieved by simultaneous treatment with TSA.

Promyelocyctic leukemia zinc finger (PLZF) protein is predominantly expressed in the most primitive hematopoietic compartment and has been shown to inhibit cell cycle of hematopoietic cells (*Mol. Cell Biol.*18: 5533(1998)). PLZF is a DNA binding protein that represses transcription by interacting with either SMRT or N-CoR which recruit histone deacetylases (Guidez, et al. *Blood* 91: 2634 (1998)) and Lin, et al. *Nature* 391: 811 (1998)).

Activation of *myc* by genomic alterations resulting from chromosomal rearrangements or retroviral insertion occurs in human, rodent and avian leukemias and lymphomas. MYC proteins are expressed in proliferating cells and are down regulated upon cell-cycle withdrawal or differentiation. MYC is part of a transcriptional network that involves other factors. MYC can dimerize with a protein called MAX which can bind to DNA and to the protein MAD. MYC-MAX dimers are transcriptionally active, whereas MAD-MAX dimers repress transcription. It has been shown that MAD-MAX recruits histone deacetylases and that this interaction is the basis for the MAX-MAD repressive activity. TSA has been shown to abolish the repression. (Laherty, et al. *Cell* 89:349 - 356 (1997)).

Another co-repressor whose activity can be modulated by histone deacetylase

inhibitors is the mammalian CBF1/RBP-Jκ which switches from a transcriptional repressor to an activator upon Notch activation. In the absence of Notch activation CBF1/RBP-Jκ represses transcription by binding to SMRT/HDAC1, while Notch activation disrupts the formation of the repressor complex. TSA treatment can induce the transcription of CBF1 regulated genes. (*Genes and Dev.*, 12: 2269-2277 (1998)). Notch signaling has been shown to influence the development of primary primitive hematopoietic precursor cells in vitro (*Blood* 1998, 91: 4084-4091) and overexpression of the activated Notch1 in the 32D myeloid cell line inhibits differentiation and permits expansion of undifferentiated cells, findings consistent with the known function of Notch in Drosophila (*PNAS* 1996, 93:13014-9).

In a recent review article, it was disclosed that the HDAC inhibitor, trichostatin A (TSA) induced a variety of biological responses in cells including induction of differentiation and cell cycle arrest. (Yoshida, et al., *BioEssays* 17:42-429(1995)). Additionally various inhibitors of HDAC have been implicated in triggering terminal differentiation of malignant cells. (Richon, et al., *Proc. Natl. Acad. Aca.* USA, 95:3003-3007 (1998)).

While the literature discloses the use of HDAC inhibitors in studies to reverse the effects of HDAC and HDAC complexes in the repression of transcription, the literature does not disclose a method of using HDAC inhibitors to promote hematopoietic stem cell cycling, to promote HSC self-renewal divisions (or replication), nor to enhance maintenance of primitive cell function during gene integration over gene integration in the absence of histone deacetylase inhibitors.

In one aspect of the invention, a method of promoting self-renewal division of hematopoietic stem cells is provided, comprising obtaining a population of hematopoietic cells from a source of hematopoietic cells, wherein the obtained hematopoietic cells include a subpopulation of hematopoietic stem cells; culturing the hematopoietic cells under growth supporting conditions; exposing the cultured cells to an effective amount of a histone deacetylase inhibitor wherein self-renewal divisions of the stem cells is promoted; and obtaining a composition of the self-renewed hematopoietic stem cells. The method may also

include a further step comprising obtaining a population of enriched hematopoietic stem cells from the hematopoietic cells prior to culturing the stem cells. Preferably the method includes selection of enriched human hematopoietic stem cells from the phenotype group consisting of $CD34^+$; Thy-1^+; $CD34^+$Thy-1^+; $CD34^-$Thy-1^+; $CD34^+EM^+$; $CD34^+$Thy-1^+Lin^-; $CD34^+CD38^{lo/-}$; $CD34^+$Thy-$1^+CD38^{lo/-}$; $CD34^+$Thy-1^+EM^+; and $CD34^+$Thy-$1^+CD38^{lo/-}EM^+$. In one embodiment, the invention includes the composition of self-renewed cells produced by the method defined above.

1. In another aspect of the invention, a culture is provided, comprising isolated mammalian hematopoietic cells which include a subpopulation of engrafting cells capable of self- renewal, an effective amount of one or more histone deacetylase inhibitors, and effective amounts of growth and self-renewal supporting cytokines wherein the effective amount of the histone deacetylase inhibitor promotes self-renewal division of the engrafting cells in the culture. In one preferred embodiment, the hematopoietic cells are human. In another prefered embodiment, the histone deacetylase inhibitor is selected from the group trichostatin A, trapoxin, chlamydocin, sodium butyrate or dimethyl sulfoxide.

In a further aspect of the invention, a method of promoting self-renewal division of stem cells is provided, comprising treating a population of stem cells with an effective amount of one or more histone deacetylase inhibitors and allowing the stem cells to self-renewal. In one embodiment the stem cells are enriched hematopoietic stem cells characterized as $CD34^+$Thy-1^+.

In an additional aspect, the invention provides a method of generating transduced mammalian stem cells comprising treating stem cells in a culture with an effective amount of a histone deacetylase inhibitor, introducing a gene into the cultured stem cells using retroviral mediated transfer, and allowing transduction of the stem cells wherein the number of transduced stem cells is increased over the number of transduced stem cells exposed to substantially the same conditions but in the absence of treatment with the histone deacetylase

inhibitor. In a preferred aspect, the stem cells are hematopoietic stem cells, preferably human hematopoietic cells, and the gene is a therapeutic gene. The method is further comprised of administering an effective amount of a population of the transduced stem cells to a mammalian subject, preferably, a human subject. In one embodiment the stem cells are allogeneic or xenogeneic to the subject and in another embodiment the stem cells are autologous to the subject.

In yet a further aspect, the invention provides a method of genetically modifying stem cells by contacting a gene delivery vehicle comprising a polynucleotide with a population of stem cells cultured in the presence of an effective amount of a histone deacetylase inhibitor, and obtaining genetically modified stem cells. In a preferred embodiment, the delivery vehicle is a retroviral vector, a lentiviral vector, an adenoviral vector or a liposome delivery vehicle.

In yet a further aspect, the invention provides a method of restoring hematopoietic capability in a subject, comprising contacting a population of hematopoietic stem cells with an effective amount of a histone deacetylase inhibitor; transducing the hematopoietic stem cells by exposing the hematopoietic stem cells to a vector including a nucleic acid sequence encoding a therapeutic gene; and administering an effective amount of a population of the transuded hematopoietic stem cells to a subject wherein hematopoietic capability is restored.

In another aspect, the invention provides a method of improving engraftment of genetically modified mammalian stem cells comprising exposing stem cells in a culture to an effective amount of a histone deacetylase inhibitor, introducing a heterologous gene into the cultured stem cells, generating an increase in the number of genetically modified stem cells over that in the absence of exposure to a histone deacetylase inhibitor, and administering the modified cells to a subject. In one embodiment, the stem cells are allogeneic and in another embodiment, the stem cells are autogolous. Preferably the heterologous gene is a therapeutic gene.

A further aspect of the invention provides a method of transducing stem cells

comprising exposing a population of stem cells to an effective amount of a histone deacetylase inhibitor; transducing the stem cells with a foreign gene; and obtaining transduced cells wherein the number of transduced stem cells is increased over the number of transduced stem cells grown under substantially the same conditions but in the absence of exposure to an effective amount of a histone deacetylase inhibitor. In one embodiment the cells are transduced in culture and in a second embodiment the cells are transduced *in vivo*. In a further embodiment the stem cells are hematopoietic stem cells, particularly mice or human. In yet another embodiment, the cells are transduced with a retroviral vector derived from Moloney murine leukemia virus or murine stem cell virus.

In yet an additional aspect, the invention provides a culture comprising a population of stem cells; an effective amount of one or more histone deacetylase inhibitors; an effective amount of one or more growth supporting cytokines; and a gene delivery vehicle including a polynucleotide encoding a marker gene or therapeutic gene. In one embodiment the gene delivery vechicle is a vector derived from a retrovirus, adenovirus or adeno-associated virus.

Figure 1 illustrates the increased number of human hematopoietic progenitor cells expressing the Thy-1 antigen from a culture of MPB $CD34^+$ cells exposed to HDAC-inhibitors.

Figures 2A and 2B illustrate that a culture of human MPB $CD34^+$ cells exposed to the HDAC inhibitor chlamydocin increases total CAFC activity within the culture relative to cultures without chlamydocin. Figure 2A shows the total CAFC activity among $Thy\text{-}1^+$ cells and Figure 2B shows the total CAFC activity among cells.

Figure 3 illustrates that a larger proportion of human $CD34^+$ hematopoietic progenitor cells cultured with chlamydocin retain expression of the Thy-1 antigen throughout 4 divisions in culture (bottom) than cells cultured without HDAC-inhibitors (top).

Figure 4 illustrates that the proportion of $Thy\text{-}1^+$ cells expressing the myeloid lineage

antigen CD15 is not upregulated on human $CD34^+$ MPB cells cultured with HDAC-inhibitors.

Figures 5A, 5B and 5C illustrate the profile of Lin, Thy-1, c-kit, and Sca-1 expression among viable murine HSCs analyzed four days after growth in media alone (A) or media supported with TSA (B) or chlamydocin (C).

Figure 6 illustrates engraftment in NOD SCID marrow of MPB $CD34^+$ cells cultured in TPO, FL, and KL with 24 hour incubation with Chlamydocin.

The following abbreviations used through out the disclosure are listed herein below:

FITC = fluorescein
TPO = thrombopoietin
FL = Flt3 ligand
KL = c-kit ligand
IL = Interleukin
LIF = leukemia inhibitory factor
MPB = mobilized peripheral blood
CFSE = carboxyfluorescein-diacetate succinimidylester
HSC = hematopoietic stem cell
TSA = Trichostatin A
DMSO = dimethyl sulfoxide
FBS = fetal bovine serum
IMDM = Iscove's modified Dulbecco's medium
HDAC = histone deacetylase
HDAC-I = histone deacetylase inhibitor
CAFC = cobblestone-area-forming cell
PE = phycoerythrin
CM = culture medium
APC = allophycocyanin
EPO = erythropoietin
FN = fibronectin fragment CH296
FACS = fluorescence-activated cell sorter

As used herein the term "stem cell" includes stem cells of various cell types, such as, muscle, epithelial, neural and bone stem cells. More particularly the invention is concerned with hematopoietic stem cells. The term "hematopoietic stem cell" (HSC) refers to mammalian and avian hematopoietic stem cells and means a population of hematopoietic cells containing the engrafting potential for *in vivo* therapeutic application. Hematopoietic cells

encompass not only HSCs, but also erythrocytes, neutrophils, monocytes, platelets, mast cells, eosinophils and basophils, B and T lymphocytes and NK cells as well as the respective lineage progenitor cells. Stem cells may also be defined *in vitro* by the presence of CAFC activity. Animal models for long term engrafting potential of candidate human hematopoietic stem cell populations include the SCID-hu bone model (Kyoizumi, et al., *Blood* 79:1704 (1992); and Murray et al., *Blood* 85: 368 – 378 (1995)), the *in utero* sheep model (Zanjani, et al., *J. Clin. Invest.* 89:1179 (1992)), NOD SCID (Larochelle, et al., *Nature Medicine* 2: 1329 – 1337 (1996) and Conneally, et al., *Proc. Natl. Acad. Sci.* 94:9836 – 9841 (1997)) and primate models (Kiem, et al., *Blood* 90:4630- 4645 (1997) and Srour, et al., *J. Hematother* 1:143 – 153 (1992)). A mouse hematopoietic stem cell has been obtained in at least highly concentrated form where fewer than about 30 cells obtained from bone marrow were able to reconstitute all of the lineages of the hematopoietic system of a lethally irradiated mouse. Each assayed cell was multipotent for all hematopoietic lineages.

An engrafting cell means any cell that can lodge in a hematopoietic tissue and function to repopulate blood cell lineages. Additionally, as used in the specification and claims, the singular form "a", "an", and "the" include plural references unless the context clearly dictates otherwise. For example, a stem cell includes a plurality of cells. The term "self renewal" or "self-renewal divisions" also referred to as "replication", is defined herein to mean cell division without apparent cell differentiation or without loss of cell engrafting capacity. The term "expansion" is intended to mean allowance of progenitor cells to increase in number and differentiate from the pluripotent stem cells used to initiate the culture.

As used herein the term "cytokine" refers to any one of the numerous factors that exert a variety of effects on cells, for example, inducing growth or proliferation. The cytokines may be human in origin, or may be derived from other species when active on the cells of interest. Included within the scope of the definition are molecules having similar biological activity to wild type or purified cytokines, for example produced by recombinant means; and molecules which bind to a cytokine factor receptor and which elicit a similar cellular response as the native cytokine factor.

Non-limiting examples of cytokines which may be used alone or in combination in the practice of the invention include, but are not limited to, IL-2, IL-3, IL-6, IL-12, IL-1α, IL-11, KL, (designated interchangeably with c-kit ligand, stem cell factor (SCF), steel factor (Stl) and mast cell growth factor (MCGF)), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), TPO, LIF, FL, and MIP-1α. As combinations, fused proteins may be employed, where the two factors are fused together or a cytokine is fused to its soluble receptor. The order of fusion will not be critical so long as the two portions of the molecules act independently and provide for their biological function. A non-limiting example includes combinations of IL-3 and IL-6 (Broxmeyer, et al., *Exp. Hematol.* 18:615 (1990). The present invention also includes culture conditions in which one or more cytokines is specifically excluded from the medium. Cytokines are commercially available from many vendors such as R & D Systems Inc., (Minneapolis, MN), Genzyme Diagnostics (Cambridge, MA) and Genentech (South San Francisco, CA).

The term "culturing" refers to the propagation of cells or organisms on or in media of various kinds.

An "effective amount" of a cytokine is an amount sufficient to promote survival, growth, and/or division of stem cells, particularly hematopoietic stem cells. "Survival" is defined herein as the ability to continue to remain alive or function.

As used herein the term "mammal" includes but is not limited to humans, mice, monkeys, farm animals, sport animals, pets, and other laboratory rodents and animals. Preferably the term refers to humans.

"Histone deacetylase activity" or " the activity of a histone deacetylase protein" refers to the biochemical activity associated with histone deacetylase proteins. The biochemical activity includes binding to and optionally catalyzing the deacetylation of an acetylated histone; this may result in mediating the activity of transcriptional co-repressors such as, but

not limited to, SMRT and N-CoR. In general, compounds that inhibit HDAC have been shown to result in activation of gene expression.

An effective dose or amount of a histone deacetylase inhibitor is defined as the amount of a HDAC-I which increases the number of self-renewal divisions of stem cells within a population of cells, particularly hematopoietic cells wherein overall cell viability may or may not be reduced and as a result increases the number of stem cells available for genetic modification as compared to stem cells within a popluation of cells grown under essentially the same conditions but in the absence of HDAC-I. .

As used herein, the term "genetic modification" refers to any addition, deletion or disruption to a cell's normal nucleotides. The method of this invention is intended to encompass any genetic modification method of exogenous or foreign gene transfer (or nucleic acid sequence transfer) into stem cells (preferably into hematopoietic stem cells). The term "genetic modification" encompasses use of a gene delivery vehicle and includes but is not limited to transduction (viral mediated transfer of nucleic acid to a recipient, either *in vivo* or *in vitro*), transfection (uptake by cells of isolated nucleic acid), liposome mediated transfer and others means well known in the art.

A "therapeutic gene" is defined herein as an entire gene or only the functionally active fragment of the gene. The therapeutic gene may be capable of compensating for a deficiency in a patient that arises from a defective or absent endogenous gene. Additionally, a therapeutic gene may be one that antagonizes production or function of an infectious agent, antagonizes pathological processes, improves a host's genetic makeup, facilitates engraftment, or a stem cell's therapeutic potency.

As used herein, the term "retroviral mediated gene transfer" and "retroviral transduction" are used interchangeably.

Methods of obtaining hematopoietic cells and stem cells are well known in the art and

include isolation of the stem cells from other cells in hematopoietic tissues of the body and particularly bone marrow. Stem cells from bone marrow appear to be in the range of about 0.01 to about 0.1% of the bone marrow cells. Bone marrow cells may be obtained from ilim, sternum, tibiae, femora, spine or other bone cavities. Other non-limiting sources of hematopoietic stem cells include embryonic yolk sac, fetal liver, fetal and adult spleen, blood, including adult peripheral blood and umbilical cord blood. (To, et al., *Blood* 89:2233-2258 (1997)).

For the isolation of bone marrow an appropriate solution may be used to flush the bone, including but not limited to, salt solution, supplemented with fetal calf serum or other naturally occurring factors in conjunction with an acceptable buffer at low concentrations, generally about 5 to 25 mM. Buffers include but are not limited to HEPES, phosphate and lactate buffers. Bone marrow can also be aspirated from the bone in accordance with conventional techniques.

The manner in which stem cells may be separated or selected from other cells is not critical to this invention. Various procedures which may be employed include magnetic separation using antibody coated magnetic beads, affinity chromatography, and cytotoxic agents joined to a monoclonal antibody. Also included is the use of fluorescence activated cell sorters (FACS) wherein the cells can be separated on the basis of the level of staining of the particular antigens. These techniques are well known to those skilled in the art and reference is made to U.S. Patent Nos. 5,061,620; 5,409,8213; 5,677,136; and 5,750,397; and Yau, et al., (1990) Exp. Hematol. 18:219-222.

The order of separation is not critical to this invention. However, preferably cells are initially separated by a coarse separation followed by separation using positive and/or negative selection. In such positive selections, up to about 15%, usually not more than about 10%, preferably not more than about 5% and most preferably not more than about 1% of the total cells in the retained cell population will lack the marker used for separation. In some instances it may be desirable to directly treat a hematopoietic cell population with an effective

dose of a histone deacetylase inhibitor without first separating a subpopulation of HSCs from the hematopoietic cells.

Stem cells, which constitute only a small percentage of the total number of hematopoietic cells are characterized by both the presence of markers associated with specific epitopic sites identified by antibodies (positive selection) and the absence of certain markers as identified by the lack of binding of specific antibodies. All of the monoclonal antibodies (mAbs) that react with a particular membrane molecule are grouped together as a cluster designator (CD). Lin^- cells refer to a cell population selected on the basis of the lack of expression of at least one lineage specific marker. For example, those human cells which lack markers associated with T cells (such as CD2, CD3, CD4, and CD8), B cells (such as CD10, CD19 and CD20), myeloid cells (such as CD 14, CD15 and CD33), natural killer cells (such as CD2, and CD56), and the like.

Human stem cells may be characterized by the following non-limiting phenotypes: $CD2^-$, $CD3^-$, $CD4^-$, $CD7^-$, $CD8^-$, $CD10^-$, $CD14^-$, $CD15^-$, $CD19^-$, $CD20^-$, $CD33^-$, $CD34^-$, $CD34^+$, $CD38^{lo/-}$, $CD45RA^-$, $CD59^{+/-}$, $CD71^-$, $CDW109^+$, $glycophorin^-$, $Thy\text{-}1^+$(CD90), $HLA\text{-}DR^{+/-}$, $AC133^+$, rhodamine 123 ($rho123^{lo}$) or a combination thereof. Monoclonal antibodies to the molecule Thy-1 in combination with mAbs to CD34 have been used to isolate murine, non-human primate and human HSCs. USP 4,714,680 describes a population of cells expressing the CD34 marker. More recently other mAbs have been identified for positive selection of human stem cells. These are designated EM, specifically, EM5, EM10 and EM16 (Chen, et al., *Immunological Reviews*, 157: 41 – 51, 1997). As used herein EM^+ means an EM phenotype that expresses any EM marker.

In one preferred embodiment the surface antigen expression profile of an enriched hematopoietic stem cell population will be selected from the following group, $CD34^+$; $Thy\text{-}1^+$; $CD10^-$; $CD19^-$; $CD15^-$; $CD33^-$; $CD34^+Thy\text{-}1^+$; $CD34^-Thy\text{-}1^+$; $CD34^+EM^+$; $CD34^+CD38^{lo/-}$; $CD34^+Rho^{123}$; $CD33^-Thy\text{-}1^+$; $CD19^-Thy\text{-}1^+$; $CD34^+Thy\text{-}1^+Lin^-$; $CD34^+Thy\text{-}1^+EM^+$;

$CD34^+CD45RA^-CD71^-$; $HLA\text{-}DR^{+/-}CD34^+$ $Thy\text{-}1^+Lin^-$; $CD34^+Thy\text{-}1^+CD38^{lo/-}$; and $CD34^+Thy\text{-}1^+CD38^{lo/-}EM^+$. In a more preferred embodiment the expression profile of an enriched population will be selected from $CD34^+$; $Thy\text{-}1^+$; $CD34^+Thy\text{-}1^+$; $CD34^-Thy\text{-}1^+$; $CD34^+EM^+$; $CD34^+CD38^{lo/-}$; $CD34^+Thy\text{-}1^+Lin^-$; $CD34^+Thy\text{-}1^+EM^+$; $CD34^+Thy\text{-}1^+CD38^{lo/-}$; and $CD34^+Thy\text{-}1^+CD38^{lo/-}EM^+$, particularly when EM^+ is $EM10^+$. The combination of markers used to isolate and define an enriched HSC population may vary as other markers become available.

Generally the number of cells obtained after positive selection will be fewer than about 20% of the original cells, more frequently fewer than about 10%, generally fewer than 5%, most frequently fewer than 1.0%, and may be as low as 0.2% or less. Compositions having greater than 80%, usually greater than about 90% of human stem cells may be achieved by the enumerated separation techniques when the desired stem cells are identified by being $CD34^+Thy\text{-}1^+Lin^-$.

Murine (m)HSCs are included in the $Lin^{neg/lo}$ population which corresponds to cells that express at the most dim levels of a lineage specific marker. For example those cells which lack expression of, or express low levels of T lymphocytes markers (such as CD2, CD3, CD4, CD5 or CD8), B lymphocytes markers (such as CD19 or B220), myeloid cells markers (such as Gr-1 or Mac-1=CD11b), natural killer markers (such as NK1.1) or erythroid markers (such as Ter119).

Murine HSCs may be characterized by the following non-limiting phenotypes: $CD2^-$; $CD3^-$; $CD4^-$; $CD4^{low}$; $CD5^-$; $CD8^-$; $CD19^-$; $B220^-$; $Gr\text{-}1^-$; $Mac\text{-}1^-$; $Mac\text{-}1^{lo}$; $NK1.1^-$; $Ter119^-$; $Ter119^{lo}$; $Sca\text{-}1^+$;$Thy1.1^{lo}$; c-Kit^- or c-Kit^{bright}; $CD34^-$; $CD34^+$; $CD38^{high}$; $CD43^{high}$; $H\text{-}2K^{high}$; or $AA4.1^+$. The combined use of monoclonal antibodies and fluorescent vital dyes such as the mitochondria-binding dye Rhodamine 123 (Rho-123) or the DNA-binding dye

Hoechst 33342 can increase the enrichment of HSC in a sorted population. The most primitive cells are enriched in the Rho-123^{lo} fraction as well as in the Hoechst 33342^{lo} fraction.

In a preferred embodiment, an enriched murine HSC population is characterized by Thy-1^{lo}; Lin$^{-/lo}$; Sca-1^{+}; and c-Kit^{+}. In a more preferred embodiment the enriched population is sorted by or selected from the phenotypes Thy-1^{low}Lin$^{-/lo}$ Sca-1^{+}; c-Kit^{+}Lin$^{-/lo}$Sca-1^{+}; c-Kit^{+}Sca-1^{+} or c-Kit^{+}Thy-1^{low}Lin$^{-/lo}$Sca-1^{+}.

In mice HSC can be largely separated, *in vitro* or *in vivo*, from more mature progenitors by their differential sensitivity to cytotoxic drugs such as 5-fluoruracil. Further separation of murine HSC can be achieved using counterflow centrifugal elutriation (CCE) on the basis of cell size and density; the small cell subset obtained by CCE at a flow rate of 25mL/min contains HSC with long term repopulating activity and very few progenitors (Jones, et al. *Nature* 347:188 (1990)).

Once hematopoietic cells are harvested, and HSCs optionally separated, the cells are cultured in a suitable medium under growth supportive conditions which includes a combination of growth factors that are sufficient to maintain the growth of hematopoietic cells. Any suitable container, flask, chamber, bag, bioreactor with perfusion or appropriate vessel such as a 24 well plate or the like can be used. Culture containers are readily available from commercial vendors. While the seeding level is not critical and will depend on the type of cells used, in general the seeding level will be at least about 10 cells per ml, more usually at least about 100 cells per ml and generally not more than about 2 x 10^6 cells per ml and usually not more than 10^5 cells per ml when the cells are CD34^{+}.

Various culture media can be used and non-limiting examples are Iscove's modified Dulbecco medium (IMDM), X-vivo 15 (serum free), and RPMI-1640. These are commercially available from various vendors for example JRH BioSciences. The formulations can be supplemented with a variety of different nutrients, growth factors, cytokines and the like. The medium can be serum free or supplemented with suitable amounts of serum such as

fetal calf serum or autologous serum or plasma. Preferably, if the cells or cellular products are to be used in humans, the medium will be serum free or supplemented with autologous serum or plasma. In general, the medium will include effective amounts of cytokines, particularly TPO, KL, FL and ILs. One skilled in the art is aware of the various growth supporting culture media and culturing techniques for HSC's and reference is made to Lansdorp, et al., *J. Exp. Med.* 175:1501 (1992) and Petzer, et al., *PNAS* 93:1470 (1996).

In one aspect the medium formulation is supplemented with TPO. A preferred concentration range is from about 0.1 ng/mL to about 500 μg/mL, more preferred is from about 1.0 ng/mL to about 1000 ng/mL, even more preferred is from about 5.0 ng/mL to about 300 ng/mL, and most preferred is from about 10.0 ng/mL to about 100 ng/mL. Additionally, the medium will include Flt3 ligand (FL) and c-kit ligand (KL) each individually at a preferred concentration range from about 0.1 ng/mL to about 1000 ng/mL, more preferred from about 1.0 ng/mL to about 500 ng/mL, and even more preferred from about 10 ng/mL to about 300 ng/mL.

While various interleukins may be used to supplement the medium, IL-6 is preferred. A preferred concentration range is from about 0.1 ng/mL to about 500 ng/mL, more preferred from about 1.0 ng/mL to about 100 ng/mL, and most preferred from about 5 ng/mL to about 50 ng/mL. Hyper IL-6, a high affinity or covalent complex of IL-6 and soluble IL-6 receptor, may also be used and reference is made to Conneally, et al., Ann. Meeting of the International Soc. for Exper. Hematology, Vancouver, Abst. #60 (1998).

Other cytokines may be added individually or in combination and include but are not limited to IL-1, IL-2, IL-3, IL-6, IL-12, IL-11, stem cell factor, G-CSF, GM-CSF, Stl , MCGF, LIF and MIP-1α. When murine stems cells are cultured, a preferred non-limiting medium includes mIL-3, mIL-6 and mSCF. A preferred concentration range is from about 0.1 ng/mL to about 1000 ng/mL, more preferred is from about 1.0 ng/mL to about 500 ng/mL, and most preferred is about 5.0 ng/mL to about 200 ng/mL.

In one embodiment, the cytokines are added to the media and replenished by media perfusion. Alternatively the cytokines may be added separately, without media perfusion, as a concentrated solution through separate inlet ports, for example when a bioreactor system is used.

Other molecules can be added to the culture media, for instance, adhesion molecules, such as fibronectin or RetroNectin™ (commercially produced by Takara Shuzo Co., Otsu Shigi, Japan). The term fibronectin refers to a glycoprotein that is found throughout the body and its concentration is particularly high in connective tissues where it forms a complex with collagen. RetroNectin™ may be used at a concentration range from about 0.1 µg/ml to about 1000 µg/ml. A more prefered range is about 0.2 µg/ml to 500 µg/ml and a most prefered range is about 0.4 µg/ml to 100 µg/ml.

HDAC-I compounds are also included in the culture medium and these compounds may be added individually or in combination, either prior to gene delivery or during gene delivery in an amount and under suitable conditions to allow stem cell self-renewal divisions. The following compounds are representative examples of HDAC-I that may be employed in the present invention.

The microbial metabolite known as trichostatin A (TSA) is a HDAC-I (Yoshida, et al., *BioEssays*, 17:42 - 429 (1995)). See EP 0 331 524 B1 for methods of preparation of TSA. Other compounds related to TSA include butyrate, particularly sodium n-butyrate; and hybrid polar compounds (HPCs), such as suberoylanilide hydroxamic acid (SAHA) and *m*-carboxycinnamic acid bishydroxamide (CBHA). These compounds have two polar groups separated by an apolar 5- to 6- carbon methylene chain. Additionally the inhibitors appear to cause the accumulation of acetylated histone H4 in cells in culture. (Richon et al., *Proc. Natl. Acad. Sci.* 95:3003 - 3007 (1998)).

Another known microbial metabolite which functions as a HDAC-I is trapoxin, a microbially derived cyclic tetrapeptide containing two L-phenylalanines, specifically Trapoxin A (TPX). (See Yoshida, et al., *BioEssays*, 17:42 - 429 (1995)). Not only is the chemical

structure of TPX completely different from TSA, the mode of inhibition is totally different from TSA and butyrate.

Cyclic tetrapeptide related compounds to TPX having the amino acid - 2-amino-8-oxo-9, 10-epoxy-decanoic acid in their molecules are included as histone deacetylase inhibitors. The following compounds are mentioned: chlamydocin (Closse, et al., *Helv. Chim. Acta* 57: 533 – 545 (1974)), HC-toxin (Liesch, et al., *Tetrahedron* 38:45 – 48 (1982)); Cyl-2; and WF-3161 (Umehara, K. J. *Antibiot* 36: 478 – 483 (1983). The fungal metabolite depudecin, (See Kwon, et al., *Proc. Natl. Acad. Sci. USA*, 95:3356 - 3361 (1998); and radicicol are also mentioned as histone deacetylase inhibitors. WO97/35990 discloses many histone deacetylase inhibitor compounds and these are incorporated by reference.

In a preferred embodiment, the HDAC-I is selected from the group of TSA, TPX, chlamydocin, butyrate, particularly sodium n-butyrate, DMSO and HDAC-I analogs thereof. While the concentration range of the HDAC-I used will vary and will depend on the specific inhibitor, a preferred concentration range will be from about 0.001 nM to about 10 mM, more preferably from about 0.01 nM to about 1000 nM.

In a preferred embodiment, the histone deactylase inhibitor is (1) TSA provided at a concentration range from about 0.01 ng/mL to 1000 ng/mL, more preferably from about 0.1 ng/mL to about 100 ng/mL, and most preferably from about 1.0 ng/mL to about 10 ng/mL, (2) TPX, provided at a concentration range from about 0.001 nM to about 100 nM, more preferably from about 0.01 nM to about 50 nM, and most preferably from about 0.1 nM to about 1.0 nM; and (3) chlamydocin, provided at a concentration range from about 0.001 nM to about 100 nM, more preferably from about 0.01 nM to about 50 nM, and most preferably from about 0.1 nM to about 1.0 nM.

While specific histone deacetylase inhibitors have been defined and numerated herein, the scope of the invention includes a broad class of histone deacetylase inhibitors including compounds not enumerated herein. One means of determining whether a compound not

enumerated herein falls within the class of histone deacetylase inhibitors includes but is not limited to using standard enzymatic assays derived from measuring the ability of an agent to inhibit catalytic conversion of a substance by the subject protein. In this manner, inhibitors of the enzymatic activity of histone deacetylase proteins can be identified. (See Yoshida, et al., *J Biol Chem.* 265:17174-17179 (1990)). Compounds may be isolated by screening for detransforming activity using oncogene-transformed cells (Kwon, et al., *Proc. Natl. Acad. Sci. USA*, 95:3356 - 3361 (1998). Furthermore, reference is made to Waterborg, et al., *Analytical Biochem.* 122:313-318 (1982) for an assay that measures acetate released by deacetylation of lysine from a peptide derived from a N-terminal sequence of histone H4. This assay involves the extraction of tritiated acetyl groups with organic solvent. A more recent reference for HDAC assays is Kolle, et al., "Biochemical Methods for Analysis of Histone Deacetylases", *Methods: A Companion to Methods of Enzymology* 15:323-331 (1998). Reference is also made to WO97/35990 for disclosure of an assay used to determine if a compound is an HDAC-I. Inhibitors of HDAC may function by different mechanisms and mention is made of TSA and TPX. In one embodiment of the invention, an effective amount of a HDAC-I is used to promote self-renewal division of stem cells and particularly hematopoietic stem cells in culture as compared to stem cells cultured under essentially the same conditions, but in the absence of HDAC-I in the culture. "Grown or cultured under essentially the same conditions" means the cells are exposed to the same concentration of growth supporting factors, the same culture medium, and the same culture period.

Once the cells are exposed to HDAC-Is, the culture period will vary and will depend on the culture conditions. Under the appropriate conditions cells potentially could be cultured indefinitely. However, in general, cells will be cultured from about 1 to about 28 days, preferably from about 1 to about 14 days, more preferably from about 1 to about 7 days. However, cells may be exposed for 1 to about 5 days or for 1 to about 3 days. Mention is made that the culture period could be less than 1 day. While not meant to be a limitation of the invention, it takes in general cultured stem cells approximately 18 to 24 hours to undergo self-renewal divisions. However, after 5 days some of the cultured cells may still be in the first division. Therefore, cultured cells after 5 days may have completed various numbers of self-

renewal divisions ranging from 1 to 7. After 5 days in culture the cells may divide at the approximate rate of one division per day or may return to a quiescent state. In one embodiment, the cells will be cultured for about 1 to about 5 days prior to exposure to an effective amount of a HDAC-I. However, the HDAC-I may be added upon the initial culturing. During this time frame, a gene delivery vehicle such as a vector comprising a nucleic acid sequence encoding a therapeutic gene or a marker gene may be introduced into the culture by means well known in the art.

Ex vivo stem cell self-renewal may be measured by various different means. Three general ways include *in vivo* assays; *in vitro* assays; and analysis of surface antigens. In vivo assays include the use of the SCID-hu mouse system. (McCune, et al., *Science* 241: 1632 – 1639, 1988). In this system, human fetal bone fragments are implanted subcutaneously into severe combined immune deficient (SCID) mice. Once the implanted human fetal bone fragments are vascularized in the SCID-hu mouse, the marrow cavity can be injected with a test human cell population and analyzed for multilineage engraftment of the donor cells. Recently other models have been developed using the beige/nude/XID(bnx) mouse and the nonobese diabetic SCID (NOD-SCID) mouse. (Greiner, et al., *Stem Cells* 16:166-177 (1998) and Nolta et al., *Proc. Natl. Acad. Sci. USA* 93:2414-2419 (1996)). The preferred way to evaluate mammalian non-human stem cell activity is by transplantation into irradiated hosts by means well known to those skilled in the art.

In vitro systems for measurement of mammalian stem cell activity includes the long term culture initiating cell assay (LTCIC) and the cobblestone-area-forming cell (CAFC) assay. (Pettengell, et al., *Blood* 84:3653 (1994); Breems, et al., *Leukemia* 8:1095 (1994); Reading, et al., *Exp. Hem.* 22:786 (Abst # 406) (1994); and Ploemacher, et al., *Blood* 74:2755 (1989)). In the CAFC assay a sparsely plated cell population is simply tested for its ability to form distinct clonal outgrowths (or cobblestone areas) on a stromal cell monolayer over a period of time. This assay gives frequency readouts that correlate with LTCIC and are predictive of engraftment in *in vivo* assays and patients. A particularly preferred CAFC assay is described in Young, et al., *Blood* 88:1619 (1996).

Flow cytometry can be used to subset hematopoietic cells from various tissue sources by the surface antigens they express. A combination of these assays may be used to test for HSCs. It is preferred that the SCID-hu mouse model and the CAFC assay be used to confirm cells with stem cell function.

In one embodiment the invention concerns a model for measuring self-renewal *in vitro* which includes labeling hematopoietic cell populations particularly enriched populations of HSC with a division tracking dye. Division tracking dyes are fluorescently tagged molecules that bind stably to subcellular structures without apparent interference with cellular function. With each cell division, fluorescence intensity of the daughter cells is halved relative to the parent cell, allowing cell division to be tracked by flow cytometry. Many dyes may be used including but not limited to carboxyfluorescein diacetate succinimidyl ester (CFSE); PKH26 (Young, et al., *Blood* 88:1619 (1996)); and PKH2 (Nordon, et al., *British J. Hematol.* 98:528 – 539 (1997) and Traycoff, et al., *Exp. Hematolo.* 26:53-62 (1998)). Most preferred is CFSE. Antibodies conjugated to other fluorochromes and directed against surface antigens characteristic of HSCs are then used to allow simultaneous measurement of cell division and primitive phenotypes.

To test stem cell function after each division cycle *in vitro*, individual populations can be purified by flow cytometry and SCID-hu, and CAFC assays can be performed on each subset. The above list of assays used to measure functional compositions of hematopoietic cell populations is not meant to be limiting in any manner. One skilled in the art may use other known assays or combinations thereof.

In another preferred embodiment, the invention concerns a method of genetically modifying stem cells including contacting a population of stem cells with a gene delivery vehicle including a polynucleotide in the presence of an effective amount of a HDAC-I. Gene delivery or transfer can be mediated by methods well known in the art including viral mediated transfer of DNA or RNA; liposome mediated transfer; and other methods as mentioned

below. The term polynucleotide should be understood to include equivalent terms such as nucleotide sequence, nucleic acids and the like.

Retroviral vectors are partivcularly prefered gene delivery vehicles. Retroviral vectors enter the cell via its normal mechanism of infection or they can be modified such that the vector binds to a different cell surface receptor or ligand to enter the cell. Additionally, recombinant derived adenovirus (AD) vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have been constructed (see WO95/00655 and WO95/11984). ADs are a relatively well characterized homogenous group of viruses including over 50 stereotypes (WO95/27071; and Frey, et al., 1998 *Blood* 8:2781-2792). ADs are easy to grow and do not require integration into the host cell genome.

Adeno-associated virus (AAV) has also been used as a gene delivery or transfer system. (U.S. Pat. No. 5,693,531 and U.S. Pat. No. 5,691,176). They are small, single-stranded DNA viruses that can integrate into the genome of infected cells. Recombinant AVV vectors have been produced in high titers which can transduce target cells at high efficiency. Herpes simplex virus (HSV) vectors are being considered by researchers for gene therapy in the transfer of gene to neural tissues. Various commercially available vectors include: pSG, pSV2CAT, and pXt1 available from Stratagene and pMSG, pSVL, and pSVK3 available from Pharmacia.

Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA in vitro or in vivo, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or in vitro transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the vectors to eliminate potentially inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression. Other non-limiting elements which

may be added to the vectors are enhancer elements, scaffold attachment regions (SAR) and matrix attachment regions (MAR), (See WO97/46687). Examples of these vectors are viruses, such as baculovirus, retroviruses, bacteriophages, cosmids, plasmids, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts and may be used for gene therapy as well as for simple protein expression.

As mentioned above, preferred vectors are retroviral vectors, and particularly preferred are amphotropic retroviral vectors. These vectors may include a therapeutic gene or a marker gene and a polynucleotide comprising the retroviral genome of part thereof. Reference is made to Coffin, et al., "Retroviruses", (1997) Chapter 9 pp: 437-473 Cold Springs Harbor Laboratory Press. Retroviral vectors useful in the methods of this invention are produced recombinantly by procedures already taught in the art. WO94/29438, WO97/21824 and WO97/21825 describe the construction of retroviral packaging plasmids and packaging cells lines. Retroviruses are subdivided into seven groups. Five of these groups represent retroviruses with oncogenic potential and the other two groups are the lentiviruses and spumaviruses. The most common retroviruses are those based on the Moloney murine leukemia virus (MoMLV-vector). Other MoMLV derived vectors include, LMiLy, LINGFER, MINGFR and MINT. Further vectors include those based on Gibbon ape leukemia virus (GALV); Moloney murine sarcoma virus (MoMSV); myeloproliferative sarcoma virus (MPSV); murine embryonic stem cell virus (MESV), for example MESV-MiLy; murine stem cell virus (MSCV); and spleen focus forming virus (SFFV). (Agarwal et al., J. of Virology, 72:3720 (1998)). Non-limiting examples of lentiviral derived vectors include vectors based on human immunodeficiency virus (HIV-1 and HIV-2). New vector systems are continually being developed to take advantage of particular properties of parent retroviruses such as host range, usage of alternative cell surface receptors and the like. Particularly preferred vectors include DNA from a murine virus corresponding to two long terminal repeats and a package signal. In one embodiment the murine viral vector is derived from a MoMLV or a MSCV. Optionally, the vector will include one or more SAR elements. However, the present invention is not limited to a particular retroviral vector, but includes

any retroviral vector wherein transduction of a population of HSCs is enhanced in the presence of HDAC-I over transduction of a population of HSCs under essentially the same conditions but in the absence of HDAC-I in the culture media.

In producing retroviral vectors constructs, the viral gag, pol and env sequence will generally be removed from the virus, creating room for insertion of foreign or heterologous DNA sequences. Genes encoded by foreign DNA are usually expressed under the control of a strong viral promoter in the long terminal repeat (LTR). Selection of appropriate control regulatory sequences is dependent on the host cell used and selection is within the skill of one in the art. Numerous promoters are known in addition to the promoter of the LTR. Non-limiting examples include the phage lamda PL promoter, the human cytomegalovirus (CMV) immediate early promoter; the U3 region promoter of the Moloney Murine Sarcoma Virus (MMSV), Rous Sarcoma Virus (RSV), or Spleen Focus Forming Virus (SFFV); Granzyme A promoter; CD34 promoter; and the CD8 promoter. Additionally, inducible or multiple control elements may be used. Plasmids containing retroviral genomes are widely available from the American Type Culture Collection (ATCC) and other sources known to those in the art.

Such a construct can be packaged into viral particles efficiently if the gag, pol and env functions are provided in trans by a packaging cell line. Therefore, when the vector construct is introduced into the packaging cell, the gag-pol and env proteins produced by the cell, assemble with the vector RNA to produce infectious virions that are secreted into the culture medium. The virus thus produced can infect and integrate into the DNA of the target cell, but does not produce infectious viral particles since it is lacking essential packaging sequences. Most of the packaging cell lines currently in use have been transfected with separate plasmids, each containing one of the necessary coding sequences so that multiple recombination events are necessary before a replication competent virus can be produced. Alternatively, the packaging cell line harbors a provirus. (The DNA form of the reverse-transcribed RNA once it integrates into the genomic DNA of the infected cell). The provirus has been crippled so that although it may produce all the proteins required to assemble infectious viruses, its own RNA can not be packaged into virus. RNA produced from the recombinant virus is packaged

instead. Therefore, the virus stock released from the packaging cells contains only recombinant virus.

The range of host cells that may be infected by a retrovirus or retroviral vector is determined by the viral envelope protein. The recombinant virus can be used to infect virtually any other cell type recognized by the env protein provided by the packaging cell, resulting in the integration of the viral genome in the transduced cell and the stable incorporation of the foreign gene product. In general, murine ecotropic env of MoMLV allows infection of rodents cells, whereas amphotropic env allows infection of rodent, avian and some primate cells including human cells. Amphotropic packaging cell lines for use with MoMLV systems are known in the art and are commercially available. Packaging cell lines include but are not limited to, PA12, PE501, PA317, PG13, ΨCRIP, RD114, GP7C-tTA-G10, ProPak-A (PPA-6), PT67and FLYA13. (See Miller, et al., (1985) *Mol. Cell Biol.* 5:431-437; Miller, et al., (1986) *Mol. Cell Biol.* 6:2895-2902; Miller et al., (1989) Biotechniques 7:980; Danos, et al. (1988) *Proc. Natl. Acad. Sci.* USA 85:6460-6464; Rigg et al., (1996) *Virology* 218:290 - 295; and Finer et al., (1994) *Blood* 83:43 – 50. Also reference is made to WO 97/21825 which discloses a method for obtaining a retroviral packaging cell capable of producing retroviral vectors, particularly supernatants produced from the packaging cell lines ProPak are taught. Recently, the G-glycoprotein from vesicular stomatitis virus (VSV-G) has been substituted for the MoMLV env protein. (See Burns, et al., (1993) *Proc. Natl. Acad. Sci* USA 90:8033-8037; and WO92/14829). Xenotropic vector systems also exist which allow infection of human cells.

The vectors will contain at least one and preferably two heterologous genes or gene sequences; (i) a marker gene and (ii) a therapeutic gene to be transferred. The following list of potential genes that may be incorporated into the vectors is given by way of example and is not meant to limit the invention in any manner.

A marker gene may be included in the vector for the purposes of monitoring successful transduction and for selection of cells into which the DNA has been integrated. Non-limiting

examples of marker genes include antibiotic resistance markers, such as resistance to G418 or hygromycin. Additionally, negative selection may be used, for example, wherein the marker is the HSV-tk gene. This gene will make the cells sensitive to agents such as acyclovir and gancyclovir. Selection could also be made by use of a stable cell surface marker to select for transgene expressing stem cells by FACS sorting. The NeoR (neomycin/G418 resistance) gene is commonly used but any convenient marker gene whose sequences are not already present in the recipient cell can be used. Further, non-limiting examples include NGFR (nerve growth factor receptor), GFP (the bacterial green fluorescent protein), DHFR (a dihydrofolate reductase gene which confers resistance to methotrexate), the bacterial hisD gene, murine CD24 (HSA), murine CD8a (lyt), bacterial genes which confer resistance to puromycin or phleomycin, and β-galactosidase.

The therapeutic gene may include genes or gene sequences effective in the treatment of adenosine deaminase deficiency (ADA); sickle cell anemia; recombinase deficiency; recombinase regulatory gene deficiency; HIV such as an antisense or trans-dominant REV gene or a gene carrying a herpes simplex virus thymidine kinase (HSV-tk)). The therapeutic gene may include gene sequences expressing the LDL (low–density lipoprotein) receptor. The therapeutic gene may also encode new antigens or drug resistant genes. Further, the therapeutic gene may encode a toxin or an apoptosis inducer effective to specifically kill cancerous cells, or a specific suicide gene to cancerous hematopoietic cells may be included. Therapeutic genes also encompass antisense oligonucleotides or ribozyme genes useful for translational suppression. The vector will usually comprise one therapeutic gene. However, more than one gene may be necessary for the treatment of a particular disease, and the vectors used in the present invention may include more than one gene. Alternatively, more than one gene can be delivered using several compatible vectors. Depending on the genetic defect, the therapeutic gene can include regulatory and untranslated sequences. For human patients, the therapeutic gene will generally be of human origin, although genes of closely related species that exhibit high homology and biologically identical or equivalent function in humans may be used if the gene does not produce an adverse immune reaction in the recipient.

Nucleotide sequences for the therapeutic gene will generally be known in the art or can be obtained from various sequence databases such as GenBank. One skilled in the art will readily recognize that any therapeutic gene can be excised as a compatible restriction fragment and placed in a vector in such a manner as to allow proper expression of the therapeutic gene in hematopoietic cells.

Methods of transduction include direct co-culture of cells with producer cells (Bregni, et al., (1992) *Blood* 80:1418-1422) or culturing with viral supernatant alone with or without appropriate growth factors and polycations (Xu, et al., *Exp. Hemat.* 22:223-230). After viral transduction, the presence of the viral vector in the transduced stem cells or their progeny may be verified by methods such as PCR. These techniques are well known in the art.

The application of gene therapy using HSC is well known and the following provides a non-exhaustive list of diseases for which gene transfer into HSCs is potentially useful. These diseases include bone marrow disorders, erythroid cell defects, metabolic disorders and the like. Particularly bone marrow transplantation should be enhanced by the methods claimed herein.

Genetically modified cells obtained by the methods described herein may be further used in autologous, xenogeneic or allogenic settings. Autologous cells are derived from an individuals own tissue; xenogeneic cells are derived from a different species, and allogenic cells are derived from a genetically different individual of the same species. The modified cells may be administered in any physiologically acceptable vehicle, normally intravascularly, although they may also be introduced into bone or other convenient site which cells may find an appropriate site for regeneration and differentiation. Usually at least 1×10^5 cells may be administered. Preferably, at least 1×10^6 or more cells may be introduced by injection, catheter or the like. If desired, factors such as TPO, IL-2, IL-3, IL-6, IL-11, GM-CSF, G-CSF, interferons, EPO and the life may also be included.

The methods provided by the present invention overcome deficiencies of prior art

methods of gene transfer by enhancing the transfer of genes into stem cells. Gene integration into stem cells by using vectors, particularly retroviral vectors requires cell division. In general, stem cell division results in differentiation. It is believed that HDAC-Is may inhibit differentiation during cell division, thereby increasing the $CD34^{+}Thy\text{-}1^{+}$ cell content of the transduced product.

The cells obtained as described above may be used immediately, expanded by means known in the art, or frozen at liquid nitrogen temperatures and stored for long periods of time, being thawed and capable of being used. The cells will usually be stored in 10% DMSO, 50% FCS, and 40% RPMI 1640 medium. Once thawed, the cells may be further expanded. Methods of expansion of HSCs by use of growth factors and/or stromal cells associated with stem cell proliferation and differentiation are well known to those skilled in the art. (US Pat. No. 5,744,361)

The stem cell composition produced by the methods herein disclosed may be used for a variety of therapeutic means as disclosed in the literature. They can be used to fully reconstitute an immuno-compromised host such as an irradiated host and/or a host subject to chemotherapy. The cells may also be used for the treatment of genetic disease. The cells may be used in bone marrow transplants, where the cells may be freed of neoplastic cells or other cells that are pathogenic. The use of stem cells will minimize graft-versus-host disease, and may also be used to induce donor-specific immunlogic tolerance in the host. Therefore an embodiment of the invention includes a method of improving engraftment of genetically modified mammalian hematopoietic cells, particularly stem cells, including exposing the hematopoietic cells in a culture to an effective amount of a HDAC-I, introducing a polynulceotide encoding a therapeutic gene or a marker gene into the cultured cells, generating an increase in the number of genetically modified cells over that in the absence of exposure to the HDAC-I and administering an effective amount of the population of modified cells to a subject. Preferably the mammal and subject are human, and the cells are enriched hematopoietic stem cells, particularly $CD34^{+}Thy\text{-}1^{+}$ cells. In a preferred embodiment, the cells are transduced with a retroviral vector selected from MoMLV and MSCV derived

vectors and optionally include one or more SAR elements.

Additionally, a method of restoring hematopoietic capability in a subject is claimed comprising contacting a population of hematopoietic stem cells with an effective amount of a HDAC-I, transducing the HSCs and administering an effective amount of a population of the transduced cells to the subject.

The practice of the present invention will employ, unless otherwise indicated conventional techniques of cell biology, molecular biology, cell culture, immunology and the like which are in the skill of one in the art. These techniques are fully disclosed in the current literature and reference is made specifically to Sambrook, Fritsch and Maniatis eds., "Molecular Cloning A Laboratory Manual, 2nd Ed., Cold Springs Harbor Laboratory Press, 1989); the series Methods of Enzymology (Academic Press, Inc.); and Antibodies: A Laboratory Manual, Harlow et al., eds., (1987).

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications cited herein are hereby incorporated by reference in their entirety.

The invention generally described above will be more readily understood by reference to the following examples, which are hereby included merely for the purpose of illustration of certain embodiments of the present invention and are not intended to limit the invention in any way.

EXPERIMENTAL

Example 1 – human cells:

Cells:

Following informed consent, leukaphersis samples are obtained from normal donors mobilized with 7.5 or 10 μg/kg/day of G-CSF for 5-6 days. $CD34^+$ cells are enriched from leukaphersis samples at SyStemix using an Isolex 300SA or 300I (Baxter Healthcare Corp., Deerfield, Illinois).

Purification of $CD34^+Thy\text{-}1^+$ cells from fresh MPB:

To select for $CD34^+Thy\text{-}1^+$ cells by flow cytometry, anti-CD34, (PR20, SyStemix Inc., Palo Alto CA) is directly conjugated to CY-5; and anti-human Thy-1 (PR13 SyStemix Inc.) is directly conjugated to phycoerythrin (PE). Purified mouse IgG1 (Sigma, St. Louis, MO) is directly conjugated to PE or Cy5 and used for isotype controls. Isolex-selected MPB $CD34^+$ cells are resuspended at 10^7 cells/mL in the staining buffer (SB) consisting of Iscove's modified Dulbecco's medium (IMDM) without phenol red (JRH Biosciences, Lenexa, KS), 2% fetal bovine serum (FBS) (Gemini Bioproducts, Calabasas, CA), 0.1% heat inactivated human gamma-globulin (Gamimune) (Miles Inc., Elkhart, IN) and 10 mM HEPES (JRH Biosciences). Cells are stained with anti-CD34-CY5 (5 μg/mL) and anti-Thy-1-PE (25 μg/mL) or appropriate isotype controls, IgG1-CY5 and IgG1-PE, for 30 minutes at 4°C, washed and resuspended in cold SB at $5x10^6$ cells /mL. Propidium iodide (PI) (Boehringer Mannheim Biochemicals, Indianapolis, IN) is added at 1 μg/mL to detect nonviable cells. $CD34^+Thy\text{-}1^+$ and $CD34^+Thy\text{-}1^-$ cell populations are sorted on a Becton Dickinson FACStar Plus TM equipped with 5W argon laser (excitation 488nm) (Becton Dickinson, San Jose, CA). Sort regions for forward versus side scatter, PI, CD34, and Thy-1 are established to select live $CD34^+Thy\text{-}1^+$ or $CD34^+Thy\text{-}1^-$ cell populations. Sorted cell populations are reanalyzed to ensure clean separation of cell populations.

Cytokines and Cell Culture:

Recombinant human thrombopoietin (TPO) is obtained from R & D Systems,

Minneapolis, MN. Recombinant Flt3 ligand (FL) and c-kit ligand (KL) are produced at SyStemix Inc., Interleukin (IL)-6 and leukemia inhibitory factor (LIF) are obtained from Novartis Inc., Basel, Switzerland. Cells are cultured for approximately 112 hours at 2 x 10^5 cells/mL ($CD34^+$ cells) or 5 x 10^5 cells/mL ($CD34^+Thy\text{-}1^+$ cells) in 6 or 24 well flat bottom plates (Corning Costar Corp., Cambridge, MA) at 37 °C in a humidified incubator with approximately 100% saturation. Cells are cultured in culture medium (CM) [X-Vivo-15 medium, (BioWhittaker, Walkersville, MD) containing 1% bovine serum albumin (BSA) (Sigma)], supplemented with the cytokines: TPO (50 ng/mL), FL (100 ng/mL), and KL (100 ng/mL). TSA is supplied at 5 ng/mL; TPX is supplied at 0.25 nM and 0.5 nM and chlamydocin is supplied at 0.25 nM or 0.50 nM. TSA is purchased from Wako Bioproducts, Richmond VA), and stored in absolute ETOH at -20 °C at a concentration of 1mg/mL. TPX and chlamydocin are provided by Novartis Pharmaceuticals Corporation, East Hanover, NJ and stored in DMSO at -20 °C at a concentration of 1mM.

As shown in Figure 1, when $CD34^+$ cells are cultured for 5 days with growth supporting cytokines (TPO, FL and KL) and the HDAC-inhibitors TSA, TPX, or chlamydocin, there is a substantial increase in the number of $Thy\text{-}1^+$ cells over cultures which do not contain HDAC-inhibitors. Among the HDAC-inhibitors tested, chlamydocin induced the greatest increase, an average of 7 fold. These results are the average of four experiments, and the error bars show the standard deviation from the mean.

Isolation of cell populations post culture:

Post culture cells are harvested from tissue culture wells, enumerated, and stained with anti-CD34-Cy5 and anti-Thy-1-PE or the appropriate isotype controls as described above. Cell populations are isolated with a FACStar Plus flow cytometer as described above for sorting pre-culture cells. Sorted cell populations are reanalyzed to ensure that a majority of events for each population falls within each sort region, and to ensure that contamination of the $Thy\text{-}1^-$ populations by $Thy\text{-}1^+$ cells is less than 5%.

Cobblestone-area-forming cell (CAFC) assay:

Cell populations are plated at limiting dilution (from 100-0.78 cells/well, 24 wells per dilution) on pre-formed murine stromal monolayers, as described in Young et al., *Blood* 88:1619 (1996). The culture medium consists of a mixture of equal portions of RPMI and IMDM medium (JRH BioSciences, Woodland, CA), 1mM sodium pyruvate (JRH BioSciences), 5 x 10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO), 10% FBS (Hyclone, Logan, UT), and cytokines, LIF (50 ng/mL) and IL-6 (10 ng/mL). Cultures are fed at weekly intervals by replacing half of the medium, and scored at week 5 for cobblestone area formation. CAFC containing wells are scored microscopically, and their frequencies estimated statistically as described below.

A frequency readout is generated from the numbers of positive wells in each row of the CAFC assay limiting dilution series using a SAS statistical analysis program incorporating the maximum likelihood estimate method and the chi-square test of linearity (Biostatistics consulting, Palo Alto, CA). Limiting dilution readouts are considered to fit a linear model if the conservative chi-squared test of linearity was greater than 0.05. The significance of differences between CAFC frequency readouts is determined using Anova (Microsoft), where P less than 0.05 is considered to be significant.

As shown in Figure 2A, the number of Thy-1^+ cells and the total CAFC activity among the Thy-1^+ fraction of cells increased 2 to 3 fold in cultures containing chlamydocin, versus cultures without chlamydocin. Total cell numbers are the same in cultures with or without chlamydocin. However, total CAFC activity in the chlamydocin containing cultures is increased two-fold. (Figure 2B). The results indicate that stem cell replication or self-renewal, measured by Thy-1^+ phenotype as well as function is increased by exposing cells to HDAC-I during culture.

SCID-hu bone assay:

The SCID-hu bone assay as described in Luens et al., *Blood* 91:1206 (1998) is utilized. The hosts are C.B. 17 scid/scid mice implanted with human fetal bones (Average gestation age 22 weeks) at 8-10 weeks prior to use in assays. Starting donor cell populations for SCID-hu assays are CD34 selected as described above. Post-culture donor cell populations are purified by flow cytometry for expression of the Thy-1 antigen as described above. The hosts are whole body irradiated (400 rads), then injected with 5,000 or 15,000 cultured Thy-1$^+$ cells per human fetal bone graft. Eight weeks after the injection, the mice are sacrificed. Bone marrow is harvested from the grafts and stained with anti-W6/32-PE for pan human leukocyte antigen HLA class I major histocompatibility complex detection versus an appropriate FITC-conjugated antibody directed toward the HLA allo-type of the donor cells. The cells are analyzed on a FACScan (Becton Dickinson Immunocytometry System). Grafts showing a minimum of 1% donor cells are scored as positive. The engraftment rate is the number of grafts positive for donor cells out of the number of grafts injected. Table 1 illustrates that stem cell function is maintained on a per cell basis among the Thy-1$^+$ expressing cells from cultures containing chlamydocin.

TABLE 1

Thy-1$^+$ cells from cultures	Dose	Engraftment Rate
(-) CHL	5,000	1/3
	15,000	4/4
(+) CHL	5,000	2/3
	15,000	3/3

CHL is chlamydocin
Dose = cultured Thy-1$^+$ cells per human fetal bone graft.
Engraftment Rate = the number of positive grafts for donor cells out of the number of grafts injected.

NOD SCID repopulation assay:

Trafficking to and engraftment in mouse bone marrow by human hematopoietic cells injected IV is measured by the NOD SCID repopulation assay. Six to ten week old NOD SCID mice (Jackson derived, and bred at SyStemix) are irradiated with 350 rads. Human MPB cells are injected into the tail vein or orbital sinus. Thy-1$^+$ cells are not repurifed after culture for the present engraftment assays; instead, equal cell numbers from the whole culture are injected. Six weeks later the mice are sacrificed, and marrow cells from the long bones of the hind limbs are recovered. Cells are fluorescently labeled with anti-CD45-APC which detects human cells and analyzed on a FACS Calibur™. Results are illustrated in Figure 6. When cell dose is low and chlamydocin is supplied in a 24 hour incubation, the engraftment is improved.

Division tracking dye labeling:

Isolex selected CD34$^+$ cells are labeled with carboxyfluorescein-diacetate succinimidylester (CFSE) dye (Molecular Probes Inc., Eugene, OR) at a cell concentration of 3-5 x 10^6 /mL and a dye concentration of 1.25 μM in IMDM without phenol red in the dark, at room temperature, for 10 minutes. The labeling is stopped by the addition of 1/5 volume of FBS and 10 fold volume of cold CM. Cells are washed and resuspended in CM. An aliquot of dye labeled cells is fixed with 1% paraformaldehyde and stored at 4 °C to be used as a marker for the fluorescence intensity of undivided cells post-culture. Analysis is performed on a FACSCalibur (Becton Dickinson, Immunocytometry System).

As shown in Figure 3, Thy-1 expression in culture after 5 days in the presence of chlamydocin is retained after at least 4 cell divisions, whereas it is more rapidly lost with cell division in cultures without chlamydocin.

Analysis of cell phenotype post-culture:

Expression of CD34 and Thy-1 antigens is evaluated using anti-CD34-CY5 and anti-Thy-1-PE as described above. FITC-conjugated monoclonal antibodies to human CD2, CD14, CD15, CD33; and CD19 (Becton Dickinson, San Jose CA) are used to evaluate

expression of lineage antigens. Analysis is performed on a FACSCalibur.

Using the above described methods, it is determined that the expression of the surface lineage antigens CD2, CD14, and CD19 is negligible both before and after 5 day culture with or without HDAC-inhibitors. The expression of CD15 and CD33 was increased post-culture, but on a similar proportion of cells in cultures with or without HDAC-inhibitors. Figure 4 shows that the proportion of Thy-1$^+$ cells that express CD15 after 5 days of culture is approximately 30%, with or without HDAC-inhibitors. These results indicate that although expression of primitive antigens such as CD34 and Thy-1 is increased on cells in cultures containing HDAC-inhibitors, the expression of other surface antigens, such as those associated with differentiation, is not.

Example 2 - murine cells:

Isolation of the murine stem cells:

Bone marrow is harvested from 4 week old BA.1 mice (C57BL/Ka.AKR/Jsys) and enriched in Sca-1 expressing cells using a biotin-conjugated anti Sca-1 antibody (Pharmingen) and positive selection using magnetic columns (Miltenyi Biotec Inc.). The Sca-1 enriched cells are further labeled with c-Kit-APC, Thy1.1-FITC, streptavidin-Texas Red and a panel of PE conjugated-lineage (Lin) antibodies directed against Mac-1, Gr-1, B220, CD2, CD4, CD5, CD8 (all antibodies purchased from Pharmingen). Propidium iodide (PI) is added at 1 μg/ml to exclude non-viable cells. The c-Kit$^+$Thy-1^{lo}Lin$^{lo/-}$Sca-1$^+$ subset, which is highly enriched for stem cell activity (Morrison and Weissman, *Immunity*, 1:661-673, (1994)) is isolated by FACS on a Vantage sorter (Beckton Dickinson, San Jose, CA).

Culture conditions:

The sorted cells are seeded in liquid culture at a density of 5x10^4 cells/ml in Xvivo15 media (BioWhittaker) in the presence of murine interleukin (mIL)-3 (10 ng/ml), mIL-6 (10 ng/ml) and murine Stem Cell factor (mSCF) (100 ng/ml) (purchased from R&D Systems). TSA (Wako Bioproducts, Richmond VA) or chlamydocin (Novartis Pharmaceuticals) are added to the culture at concentrations ranging from 0.5 to 10 ng/ml and 0.25 to 2 nM,

respectively. The cells are cultured 4 days in a 37°C incubator with 5% CO_2 in multi-well flat bottom tissue culture plates (either 96 well, 48 or 24 well plates depending on the initial number of cells seeded).

Analysis of cells post culture:

After 4 days of culture, the cells are harvested and an aliquot is stained with Trypan Blue to determine the number of viable and non-viable cells using a Neubauer hemacytometer. At least 200 total cells are scored. A portion of the cultured cells (equivalent to at least 40 000 viable cells) ares then stained as described above. The profile of c-Kit , Thy-1, Lin and Sca-1$^+$ expression is then determined by FACS using a Vantage sorter. The limits of the gates used to determine the percentage of cells that fall into the c-Kit$^+$Thy-1$^+$Lin$^{lo/-}$Sca-1$^+$ subset is defined as the frontiers separating the bimodal population for each stain observed separately. These limits are set with cells treated with mIL-3, mIL-6 and mSCF alone. The number of c-Kit$^+$Thy-1$^+$Lin$^{lo/-}$Sca-1$^+$ cells present in the culture is calculated by multiplying the proportion of cells in the c-Kit$^+$Thy-1$^+$Lin$^{lo/-}$Sca-1$^+$ subset by the total number of viable cells present in the culture.

Results from a representative experiment are summarized in Table 2 and illustrated in Figures 5A, 5B and 5C.

TABLE 2

HDAC-I added	Viable cell ($x10^3$)	% of non viable cells	% of KTLS	Fold expansion of KTLS cells
None	1 950	16%	8%	3.1
TSA (3ng/ml)	610	6%	58%	7.2
Chlamydocin (1nM)	450	3%	57%	5.0

KTLS is c-Kit$^+$Thy-1$^+$Lin$^{lo/-}$Sca-1$^+$

In this experiment $50x10^3$ sorted c-Kit$^+$Thy-1^{lo}Lin$^{lo/-}$Sca-1$^+$(KTLS) cells are cultured in 1ml of X vivo15 media supplemented with mIL-3, mIL-6 and mSCF in a 24 well-plate. When indicated TSA (3ng/ml) or chlamydocin (1nM) is added to the culture. After 4 days, the cells are collected for cell count and FACS analysis. The number of viable cells recovered after 4 days of culture is diminished 2 to 4 fold by the addition of HDAC-I to the growth-media. Compared to cells grown in the absence of HDAC-I, the vast majority of cells treated with TSA or chlamydocin remains Lin negative (93 or 88% versus 55%) and expresses high levels of Thy-1 (95-96% versus 30%) and Sca-1 (94 or 88% versus 46%). A higher percentage of cells grown with either HDAC-I also remain positive for c-Kit compared to cells cultured without HDAC-I (67 and 73% versus 51%). While only 8% of the cultured cells retained the stem cell phenotype (c-Kit$^+$Thy-1^{lo}Lin$^{lo/-}$Sca-1$^+$) when grown in the absence of HDAC-I, these cells represent 58 and 57% of the cells cultured with TSA or chlamydocin, respectively. Over the 4 day-culture period, the c-Kit$^+$Thy-1^{lo}Lin$^{lo/-}$Sca-1$^+$cells grown without the addition of HDAC-I underwent a 3.1-fold expansion while those cultured with TSA or chlamydocin underwent a 7.2- or 5.0-fold expansion, respectively.

Figures 5A, 5B and 5C illustrate the profile of Lin, Thy1, cKit and Sca-1 which are analyzed after 4 days of culture in growth media alone (A) or media supplemented with 3 ng/ml of TSA (B) or 1nM of chlamydocin (C). The limits of the regions used to calculate the percentage of cells that fall into each gate are depicted on the dot plots. The nonviable cells are excluded from the analysis.

Example 3 - RetroNectin™ Transduction:

Retroviral Infection:

Following informed consent, as described above in example 1, the CD34$^+$ of CD34$^+$Thy-1$^+$ enriched cells are cultured at 10^6 cells per ml in 5 mL cultures in X-Vivo-15 medium (BioWhittaker, Walkersville, MD) for 48 hours at 37°C in 5% CO_2. The cultures are supplemented with the cytokines: TPO (100 ng/mL), (R&D Systems, Minneapolis, MN); KL

(100 ng/mL), and FL (100 ng/mL), SyStemix, Palo Alto CA. The histone deacetylase inhibitor, chlamydocin is supplied to the cultures at 0.50 nM per mL for 72 hours. Chlamydocin is provided by Novartis Pharmaceuticals Corporation, East Hanover, NJ and is stored in DMSO at –20 °C at a concentration of 1mM.

Non-tissue culture-treated plates (Falcon, Lincoln Park, NJ) are coated at 2 μg per cm^2 of fibronectin fragment CH-296 (FN) (BioWhittaker, Walkersville, MD) and incubated for 2 hours at 37°C. Plates are blocked with 2% human serum albumin in phosphate buffered solution (HSA/PBS) for 30 minutes. This is followed by washing with 2.5% Hepes buffer and Hanks Balanced Salt Solution (HBSS).

After 48 hours, cultured cells are centrifuged for 10 minutes at 1200 rpm at 4 °C and resuspended in the same medium as described above. The cells are added to FN-coated plates containing an equal volume of retroviral supernatant for 20 hour culture at 37°C in 5% CO_2 without polybrene or protamine sulphate. (Lishan SU et al., 1997, Hematopoietic Stem Cells-Based Gene Therapy for Acquired Immunodeficiency Syndrome: Efficient Transduction and Expansion of RevM10 in Myeloid Cells *In Vivo* and *In Vitro*. Blood:Apr. 2283 – 2290).

The Moloney murine leukemia virus (MoMLV) vector is prepared from a ProPAk (PP-A.6) packaging cell line (Forestell, et al., 1997, Novel Retroviral Packaging Cell Lines: Complementary Tropisms and Improved Vector Production For Efficient Gene Transfer, Gene Therapy, 4:600-610). The vector comprises - LTR-NGFR-SV40Neo-LTR- wherein LTR is the viral long terminal repeat; NGFR is the truncated human nerve growth factor receptor, SV40 is the SV40 promoter, and Neo encodes G418 resistance. (Miller, et al., 1989, Improved Retroviral Vectors for Gene Transfer and Expression. BioTechniques. 7:980 –990).

Cells are removed from the plates by gentle pipetting and centrifuged (as described previously). Cell pellets are resuspended in X-vivo 15 medium plus 1% BSA. Viable cells are counted by trypan blue exclusion. After diluting, at a 1:10 ratio of cells to trypan blue, cells are placed in suspension in a hemacytometer. Dead cells are then determined by their blue

color. These methods are known to those skilled in the art.

FACS analysis of gene expression on CD34$^+$, Thy-1$^+$ and total cells:

After transduction, cells are analyzed by FACS as described below. A subset of the cells as described above are placed in X-vivo 15 culture medium containing GM-CSF,10ng/ml; EPO, 2 U; IL-3, 10 ng/ml; IL-6, 10 ng/ml; LIF, 100 ng/ml; and KL 100 ng/ml. Cultures (1.0 ml) are performed in 24-well tissue culture plates (Falcon). At 72 hours, cultured cells are harvested and stained with anti-CD34-APC (Becton Dickinson, San Jose, CA), anti-Thy-1-PE (SyStemix, Palo Alto CA), and anti-NGFR-FITC (Boehringer Mannheim, Indianapolis, IN) or appropriate isotype controls (Becton Dickinson, San Jose, CA). Fluorescence is analyzed on a FACS Calibur (Becton Dickinson) using standard techniques. The anti-NGFR-FITC is conjugated at SyStemix. Results summarized in Table 3 demonstrate the application of chlamydocin increased not only the number of cells expressing Thy-1 antigen but also the number of Thy$^+$ NGFR$^+$ cells.

TABLE 3

NGFR Transgene Expression in Primitive Thy-1$^+$ MPB Cells After Short-term Culture (3 Days) With TPO, FL, and KL and with or without Chlamydocin

	%NGFR$^+$ OF THY-1$^+$		CELL#X 10E5 OF THY-1$^+$NGFR$^+$	
	AVE	SD	AVE	SD
(-) CHL	11.60	3.9	0.69	0.4
(+) CHL	11.20	3.7	1.68	0.8
CHL + BSA	12.33	5.9	1.56	0.6
Fold Increase (+) CHL			2.44	

Footnote to Table 3.

For (+) and (-) CHL each value is the average (AVE) of 5 experiments.
For CHL + BSA each value is the average of 3 experiments.
SD = standard deviation.
CHL is Chlamydocin supplied at 0.5 nM/mL.
BSA is Borine Serum Albumin and is supplied at 1.0 %.
Fold Increase = (+) average CHL / (-) average CHL

In Vitro Assay for Transduction of Primitive PHP:

After approximately 72 hours of transduction, the cells are counted and twenty thousand cells per culture condition (+ or – chlamydocin) are plated on top of SyS1 murine stromal cells (Young et al., Blood, vol. 87: 545 1996) in 2 wells in 24-well plates (Corning Science Products, Acton MA) for 5-week culture in the presence of exogenous human IL-6 (20 ng/ml) and LIF (100 ng/ml). Plates are fed weekly by exchange of half the medium volume. The medium includes a mixture of 50/50 RPMI/IMDM plus 10% fetal calf serum. After 5 weeks in stromal culture, there is no significant difference between % NGFR in $CD34^+$ cells for cultures with or without chlamydocin. (Data not shown)

FACS Analysis of Gene Expression on $CD34^+$ and total cells cultured on SYS1 Stroma:

Cells from above are harvested and filtered through 70mm cell strainers (Falcon) to remove stromal cell clumps. Viable hematopoietic cells are counted, and the cells cultured for 3 days in X Vivo 15 medium containing GM-CSF, 10 ng/ml; EPO, 2U; IL-3, 10 ng/ml; IL-6, 10 ng/ml; LIF, 100 ng/ml; and KL, 100 ng/ml. Cells are stained with anti-NGFR-FITC and anti-CD34-APC (Becton Dickinson). Cells are then analyzed on a FACS Calibur.

Further LTC-CFC Assays for SV40Neo on LTC-CFC can be performed:

Triplicates of 40,000 cells/ml from C are placed into methylcellulose colony assays (MethoCult, StemCell Technologies, Vancouver, Canada) with GM-CSF, 10 ng/ml; EPO, 2U; IL-3, 10 ng/ml; IL-6, 10 ng/ml; and KL, 100 ng/ml. Hematopoietic colonies are scored after 12 - 14 days according to standard criteria known in the art.

Individual colonies (64) are placed into 50 µl of lysis buffer as described below. Lysates are incubated overnight at 37 °C and heat inactivated at 95°C for 15 minutes, before storage at -20°C. PCR assays are performed to test transgene marking with SV40Neo. B-

globin is used as a positive control for the presence of DNA. PCR lysis buffer solutions, A and B, are mixed at a ratio of 1:1 with 144 mg Proteinase K. Lysis buffer solution A includes 100 mM KCL, 10 mM Tris HCL, and 2.5 mM $MgCl_2$. Lysis buffer solution B includes 10 mM Tris HCL, 2.5 mM $MgCl_2$, 1% Tween, and 1% NP40. The final concentration obtained is 100 mg/ml Proteinase K. For PCR analysis, 10 µl (1000 cells) and 5 µl (500 cells) aliquots are dispensed into thermocycle plates. Specific known primers and probes are used for PCR:

PCR is carried out in 30 µl volumes, containing 10 mM Tris-HCL (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCL, 0.1666 mM each of dATP, dCTP, dGTP, dTTP, 0.833 µM of SvNeo forward primer 1 and SvNeo reverse primer 2, 0.227 µM B-globin forward primer 1 and B-globin reverse primer 2, and 1 unit of Taq DNA polymerase. The PCR program can be preformed on a Perkin-Elmer 9600 thermocycler: one cycle of 95 °C for 5 minutes and 40 cycles of 95 °C for 30 seconds, 62 °C for 30 seconds and 72 °C for 1 minute, followed by one cycle of 72 °C for 10 minutes. PCR products are analyzed by gel electrophoresis on 3 % agarose gels. These techniques are well known in the art.

Example 4 - Spinoculation Transduction:

Hematopoietic stem cells are purified by selection of $CD34^+$ cells by an immune affinity system from G-CSF mobilized peripheral blood obtained from healthy volunteers using standard practices and also as described in Example 1 above.

The purified $CD34^+$ cells (3×10^6) are cultured in 7ml Teflon cell culture bags (American Fluoroseal, Inc., Gaitherburg, MD) for 48 hours in 1.5 ml serum-free X-vivo 15 medium. The size of the Teflon bag used in an experiment is dependent on the number of cells to be cultured and subsequently transduced. The medium is supplemented with hematopoietic cell growth factors including TPO, 200 ng/mL; FL, 200 ng/mL; IL-3, 40 ng/mL; IL-6, 40 ng/mL; and LIF, 200 ng/mL in the presence or absence of the histone deacetylase inhibitor, chlamydocin is supplied at 0.50 nM per mL.

After 48 hours an equal volume (1.5 ml) of retroviral supernatant is added to the cultured cells. The retroviral vector and supernatant used in this example is described above in example 3. The cells are centrifuged at 3400 rpm (Sorval RD6000) oriented with the flat side of the bag placed at the bottom of the centrifuge bucket for 4 hours. The cells are resuspended by gentle pumping of the bag, and cells are returned to the incubator. After 20 hours at 37 °C in 5% CO_2, the cells are harvested from the bag, and analyzed for stem cell content ($CD34^+$ and $CD34^+Thy\text{-}1^+$) as described above in Example 3.

TABLE 4

Cell Recovery Post Transduction

	Percent Cell Recovery			
	Without Chlamydocin		With Chlamydocin	
	Mock	Transduced	Mock	Transduced
Range	83.9 – 115.7	60.0 – 158.6	83.2 – 144.3	81.4 – 151.1
Mean	104.5	95.9	118.5	113.
StDev	12.0	37.0	25.60	25.
n =	6	6	3	6

The data shown in Table 4 indicates that cell recovery after the transduction process is not effected by chlamydocin.

TABLE 5

The Effect of Chlamydocin on Stem Cell ($CD34^+$ $Thy\text{-}1^+$) Content Post Transduction at Day 3

	% $CD34^+$ $Thy\text{-}1^+$		
	Starting (Input)	Post Transduction	
		(-) Chlamydocin	(+) Chlamydocin
Range	40.8 – 69.8	24.9 – 44.6	49 – 79.8
Mean	54.7	38.6	67.1
StDev	9.4	7.6	11.5

The mean represents the average of six experiments.

The data provided in Tables 4 and 5 are used to calculate the absolute number of HSC ($CD34^+$ $Thy\text{-}1^+$) and the results are illustrated in Table 6. Yields are per 1 x 10^6 cells at the start of transduction. The results suggest that there are approximately 2 fold more HSCs in the populations transduced with chlamydocin as compared to cells transduced without the chlamydocin. Cells transduced in the presence or absence of chlamydocin showed equivalent content of $CD34^+$ cells (99 +/-1.4%) after the 3 day transduction process.

TABLE 6

Calculation of Chlamydocin Effect on Stem Cell Content Post Transduction

	# of $CD34^+Thy\text{-}1^+$ cells	% of input
Input (starting)	547000	100
(-) Chlamydocin	370000	67.6
(+) Chlamydocin	758000	138.6

TABLE 7

Transgene Expression on HSC

	% Cells Expressing NGFR					
	Total Cells		$CD34^+$		$CD34^+Thy\text{-}1^+$	
	(-) CHLAM	(+) CHLAM	(-) CHLAM	(+) CHLAM	(-) CHLAM	(+) CHLAM
Range	8.7 – 19.3	7.4 – 28.4	8.8 – 19.8	7.6 – 29.4	5.0 –15.1	5.0 – 27.2
Mean	13.1	18.1	13.5	18.7	9.1	15.9
StDev	3.9	7	4	7.3	4	7.9

TABLE 8

Relative Effect of Chlamydocin on Thy Content and Thy$^+$ Cells That Express Transgene on Day 3 As Compared to No Chlamydocin

	Value Relative to (-) Chlamydocin	
	% CD34$^+$Thy-1$^+$	% Thy-1$^+$ expressing NGFR
Range	1.63 – 1.96	0.93 - 2.43
Mean	1.75	1.71
StDev	0.12	0.45

Footnote to Table 8: 1.75 x 1.71 = approximately 3 fold increase in the # of Thy$^+$ cells that express the transgene in the infusion product. The mean represents six experiments.

Example 5 - Dose Response For Chlamydocin:

CD34$^+$ selected cells are transduced by spinoculation with the MoMLV vector as described in Example 3. The results shown in Table 9 illustrate chlamydocin operates in a dose dependent manner in increasing the stem cell content (CD34$^+$Thy-1$^+$) of the ex vivo transduced CD34$^+$ cells derived from mobilized peripheral blood. The data indicates that the optimal concentration is between 0.5nM and 1.0nM for cells incubated for three days. Additionally, the results illustrate there is an increase in the stem cell content relative to the starting CD34$^+$ population in the presence of 0.5 nM Chlamydocin.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 5/06, C07K 14/47

A3

(11) International Publication Number: **WO 00/23567**

(43) International Publication Date: 27 April 2000 (27.04.00)

(21) International Application Number: PCT/EP99/07741

(22) International Filing Date: 14 October 1999 (14.10.99)

(30) Priority Data:

09/173,633	16 October 1998 (16.10.98)	US
60/132,476	4 May 1999 (04.05.99)	US

(71) Applicant *(for all designated States except AT US)*: NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, CH–4058 Basel (CH).

(71) Applicant *(for AT only)*: NOVARTIS–ERFINDUNGEN VERWALTUNGSGESELLSCHAFT M.B.H. [AT/AT]; Brunner Strasse 59, A–1230 Vienna (AT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: LAVAU, Catherine, P. [FR/US]; 4296–A Wilkie Way, Palo Alto, CA 94306 (US). YOUNG, Judy, Carol [US/US]; 2833 Tramanto Drive, San Carlos, CA 94070 (US). HILL, Beth, Louise [US/US]; 981 Kintyre Way, Sunnyvale, CA 94087 (US).

(74) Agent: BECKER, Konrad; Novartis AG, Corporate Intellectual Property, Patent & Trademark Department, CH–4002 Basel (CH).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
27 July 2000 (27.07.00)

(54) Title: PROMOTION OF SELF–RENEWAL AND IMPROVED GENE TRANSDUCTION OF HEMATOPOIETIC STEM CELLS BY HISTONE DEACETYLASE INHIBITORS

(57) Abstract

A method of promoting stem cell self–renewal is disclosed which comprises exposing a population of stem cells, particularly hematopoietic stem cells, to an effective dose of a histone deacetylase inhibitor, particularly trichostatin A, trapoxin, or chlamydocin. The invention is also directed to the use of histone deacetylase inhibitors to increase the number of transduced stem cells.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/EP 99/07741

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 16535 A (SANDOZ LTD ;SYSTEMIX INC (US); SANDOZ AG (DE); SANDOZ AG (AT)) 9 May 1997 (1997-05-09) the whole document	1-34
A	EP 0 827 743 A (UNIV BRUXELLES) 11 March 1998 (1998-03-11) the whole document	5,6,9,11
A	EP 0 827 742 A (UNIV BRUXELLES) 11 March 1998 (1998-03-11) the whole document	5,6,9,11
A	EP 0 627 487 A (IMMUNEX CORP) 7 December 1994 (1994-12-07) the whole document	26-33

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 April 2000	02/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ..ional Application No
PCT/EP 99/07741

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,P	LAVAU C. ET AL.: "Inhibitors of histone deacetylases promote primitive hematopoietoic cell self-renewal in ex vivo culture" BLOOD, vol. 92, 15 November 1998 (1998-11-15), pages 504a-505a, XP000904836 abstract	1-34

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...ernational application No.
PCT/EP 99/07741

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Insofar as claims 25-29 and 31 are intended as to be directed to a method of treatment of the human/animal body, then the search must be considered as been carried out and based only on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/EP 99/07741

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9716535 A	09-05-1997	AU 7495396 A	22-05-1997
		CA 2236263 A	09-05-1997
		EP 0858503 A	19-08-1998
		JP 11514879 T	21-12-1999
EP 0827743 A	11-03-1998	EP 0827742 A	11-03-1998
		JP 10114681 A	06-05-1998
		US 5993845 A	30-11-1999
EP 0827742 A	11-03-1998	EP 0827743 A	11-03-1998
		JP 10114681 A	06-05-1998
		US 5993845 A	30-11-1999
EP 0627487 A	07-12-1994	US 5554512 A	10-09-1996
		AU 683472 B	13-11-1997
		AU 6987794 A	20-12-1994
		BR 9407073 A	27-08-1996
		CA 2162397 A	08-12-1994
		CN 1125479 A	26-06-1996
		CZ 9503079 A	16-10-1996
		FI 955646 A	23-01-1996
		HU 74831 A	28-02-1997
		JP 8511251 T	26-11-1996
		NO 954735 A	23-01-1996
		NZ 267541 A	24-06-1997
		PL 311756 A	18-03-1996
		WO 9428391 A	08-12-1994
		US 5843423 A	01-12-1998
		ZA 9403490 A	23-01-1995
		AU 702179 B	18-02-1999
		AU 2098295 A	25-09-1995
		CN 1142247 A	05-02-1997
		EP 0749472 A	27-12-1996
		FI 963373 A	29-08-1996
		NO 963630 A	07-11-1996
		NZ 282999 A	19-12-1997
		WO 9524469 A	14-09-1995